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Efficient large scale purification of his-tagged proton translocating NADH:ubiquinone oxidoreductase (complex I) from the strictly aerobic yeast *Yarrowia lipolytica*

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Abstract

Proton translocating NADH:ubiquinone oxidoreductase (complex I) is the largest membrane bound multiprotein complex of the respiratory chain and the only one for which no molecular structure is available so far. Thus, information on the mechanism of this central enzyme of aerobic energy metabolism is still very limited. As a new approach to analyze complex I, we have recently established the strictly aerobic yeast *Yarrowia lipolytica* as a model system that offers a complete set of convenient genetic tools and contains a complex I that is stable after isolation. For crystallization of complex I and to obtain its molecular structure it is a prerequisite to prepare large amounts of highly pure enzyme. Here we present the construction of his-tagged complex I that for the first time allows efficient affinity purification. Our protocol recovers almost 40% of complex I present in *Yarrowia* mitochondrial membranes. Overall, 40–80 mg highly pure and homogeneous complex I can be obtained from 10 l of an overnight *Y. lipolytica* culture. After reconstitution into asolectin proteoliposomes, the purified enzyme exhibits full NADH:ubiquinone oxidoreductase activity, is fully sensitive to inhibition by quinone analogue inhibitors and capable of generating a proton-motive force.

Keywords: Mitochondria; NADH; Complex I; Yeast; Affinity purification; Proteoliposome; *Yarrowia lipolytica*

1. Introduction

Proton translocating NADH:ubiquinone oxidoreductase, also called complex I, is the first electron translocating protein complex of the mitochondrial respiratory chain. It is one of the biggest and most complicated membrane bound protein complexes known, consisting of 43 subunits in mammalian mitochondria [1,2] and of at least 35 subunits in mitochondria of fungi like the yeast *Yarrowia lipolytica* [3]. Complex I plays an essential role in oxidative phosphorylation, in which it catalyzes a two electron transfer from NADH to ubiquinone linked to the translocation of four protons across the mitochondrial membrane [4]. This reaction is the first step in generating the proton-motive force which drives ATP synthesis. Despite the physiological importance of

Abbreviations: BN-PAGE, blue-native polyacrylamide gel electrophoresis; CCCP, carbonyl cyanide *p*-chloromethoxyphenylhydrazone; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; dNADH, deaminodihydronicotinamide adenine dinucleotide; DQA, 2-decyl-4-quinazolinyl amine; HAR, hexammineruthenium(III)-chloride; DBQ, *n*-decylubiquinone; MOPS, 3-(*N*-morpholino)propanesulfonic acid; PCR, polymerase chain reaction; PMSF, phenylmethylsulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane

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complex I and the knowledge of an increasing number of diseases linked to defects in this enzyme [5,6], no mechanistic details about the electron pathway within the protein or the coupling of electron transport to vectorial proton translocation over the mitochondrial membrane are known. Little structural information is available, due to the high complexity and size of this multi-subunit enzyme. However, progress has also been hampered by the lack of a model system in which genetic manipulation can be achieved easily and which at the same time contains stable complex I that can be easily isolated to allow for a broad range of protein chemical, biophysical and structural studies.

Recently, we have established the obligate aerobic yeast *Y. lipolytica* as a model system to study mitochondrial complex I [7,8] that meets these requirements [3]. *Y. lipolytica* offers a complete set of yeast genetic tools [9], including the convenient possibility to introduce mutated gene copies on *Escherichia coli*-*Y. lipolytica* single copy shuttle plasmids.

The efficiency and success of functional and structural studies with such a large membrane bound enzyme complex are highly dependent on the availability of a convenient and fast protocol to obtain homogeneous enzyme on a large preparative scale at high yield. We have achieved this goal by deleting the chromosomal copy of the *NUGM* gene of *Y. lipolytica* and reintroducing a plasmid-borne copy with an added his tag sequence. This construct allowed for rapid affinity chromatography on a Ni-NTA column and yielded large amounts of highly pure and homogeneous complex I in a few hours.

2. Materials and methods

2.1. Strains

Y. lipolytica strains E129 (*Mat*A, *lys11*-23, *ura3*-302, *leu2*-270, *xpr2*-322) and E150 (*Mat*B, *his*-1, *ura3*-302, *leu2*-270, *xpr2*-322) were a kind gift from Prof. C. Gaillardin, INRA, Paris, France. The diploid strain GB1 was produced by mating E129 and E150. *Y. lipolytica* genetic techniques were carried out according to [9].

2.2. Deletion of NUGM, the gene coding for the 30 kDa subunit of Y. lipolytica complex I

The complete *NUGM* gene of *Y. lipolytica* (accession No. AJ249784) has been isolated and sequenced previously [3]. Clone 1C4 containing a 4.8 kb genomic DNA insert comprising the entire *NUGM* gene in pBluescript SK$^-$ was used as starting point to construct the *nugm*::*URA*3 deletion allele. First, a copy of this plasmid, excluding the *NUGM* open reading frame, was obtained by inverse PCR using primers 30-1 (5′-TGGCGAATCGAGAGAGCA-3′, antisense strand), corresponding to bases 2–19 of the *NUGM* open reading frame, and 30-2 (5′-TGGGAATGTAGGGTGAGA-3′), starting 45 bases downstream from the stop codon (Fig. 1). The 6.8 kb PCR product was blunt end ligated to a 1.6 kb PCR product containing the complete *Y. lipolytica* *URA3* gene (primers Ura1: 5′-CCCATACATTCTTCGTTGGAGG-3′, Ura2: 5′-CGCCATTGGCTTCTCTCTCTTGA-3′).

A clone in which the orientation of the *URA3* reading frame was opposite to the original *NUGM* gene was selected and a 3 kb *Sal*I fragment containing the *URA3* gene, flanked by about 700 bp of downstream and 500 bp of upstream sequence from the *NUGM* locus, was used for transformation of diploid *Y. lipolytica* GB1 cells. Sixteen *URA3* colonies were selected and screened for homologous recombination by PCR using a combination of inward primers derived from sequences outside of the 3 kb *Sal*I fragment (30ds, 30us) and outward primers derived from the *URA3* sequence (URAds, URAus) and by Southern blotting (data not shown). The diploid *Y. lipolytica* clone NK1 was identified as a heterozygous homologous recombinant carrying the *nugm*::*URA3* deletion allele and a wild-type *NUGM* allele.

2.3. Construction of an expression vector carrying the gene for a his-tagged 30 kDa subunit

A 3 kb *Sal*I fragment from the genomic clone containing the *NUGM* gene was subcloned into the *Y. lipolytica* shuttle vector pINA240 [9], bearing a *LEU2* marker gene. Codons for a his tag of six consecutive histidines with or without an additional linker of six consecutive alanines were fused to the

C-terminal end of the *NUGM* open reading frame by means of inverse PCR in two steps (Fig. 2). Plasmid pINA240/*NUGM* was used as a template for inverse PCR. The primers were designed to amplify the entire plasmid and to add the his tag codons by extending the C-terminus in the first PCR. The sense primer was P1 (5′-CAT CAT CAC TAG TTG AGA TAG AGC GAT GCT GG-3′) coding for three histidines and the stop codon, the antisense primer was P2 (5′-ATG ATG ATG CTT CTT GCT GCC TTC CTT C-3′) coding for three additional histidines. By this strategy, 20 base pairs downstream from the stop codon were deleted. The 7.8 kb PCR product was self-ligated over blunt ends as described above. This construct bearing six histidines at the C-terminus of *NUGM* was referred to as pNK1.1. In the next step, pNK1.1 was used as a template for another inverse PCR. Following the same strategy as described above, codons for a six alanine linker were introduced in front of the six histidine codons, using the sense primer P3 (5′-AGC TGC TGC CTT CTT GTC GCC TTC CTT CTC-3′) coding for three alanines and the antisense primer P4 (5′-GCC GCA GCC CAT CAT CAT CAT CAT CAC TAG-3′) coding for three additional alanines. The final plasmid resulting from self-ligation of the PCR product is referred to as pNK1.2. All constructs were verified by DNA sequencing.

2.4. Generation of *Y. lipolytica* strains containing his-tagged complex I

Plasmids pNK1.1 and pNK1.2, carrying a modified *NUGM* gene encoding the 30 kDa subunit with C-terminally added his tag and his tag/linker extensions, respectively, were transformed into the heterozygous diploid strain NK1 carrying the *nugm*::*URA3* deletion allele by the PLATE transformation method [10]. Haploid deletion strains complemented by plasmid pNK1.1 or pNK1.2 were obtained through sporulation and random spore analysis and referred to as NK1.1 and NK1.2, respectively.

Presence of the his tag was demonstrated by Western blotting of mitochondrial membranes using commercially available anti-his tag antibody (Anti-His_6 peroxidase conjugate, Sigma) and by two-dimensional blue-native gel electrophoresis [11] of mitochondrial membranes.

2.5. Measurement of catalytic activity

We used DBQ (*n*-decylubiquinone) as a substrate for the determination of dNADH:ubiquinone oxidoreductase activity. Steady state activity was recorded on a Shimadzu UV-300 spectrometer in the dual wavelength mode following dNADH oxidation at 340–400 nm ($\varepsilon = 6.10\ mM^{-1}\ cm^{-1}$) at 30°C [12].

To monitor activity during complex I preparation, dNADH oxidation was measured as described above, but the artificial electron acceptor HAR (hexaminеruthenium(III)-chloride) was used because this activity can be taken as a direct measure for complex I content [13]. 2 mM HAR and 200 μM dNADH were used in a buffer containing 20 mM Na-HEPES, pH 8.0, 250 mM sucrose, 2 mM EDTA and 2 mM NaN_3.

For measurement of complex I activity in proteoliposomes, dNADH:DBQ activity was assayed in 20 mM Na/MOPS, pH 7.2, 50 mM NaCl, 20 mM KCl, 2 mM KCN.

2.6. Affinity purification of his-tagged complex I from *Y. lipolytica*

Y. lipolytica cells were grown overnight at 27°C in a 10 l Biostat E fermenter (Braun, Melsungen) in modified YPD medium (2.5% glucose, 2% bactopeptone, 1% yeast extract) and harvested by centrifugation for 10 min at $5000 \times g$ and 4°C. Typically, 700–800 g of wet cell mass was obtained from 10 l of culture. Mitochondrial membranes were prepared from the haploid mutant strains NK1.1 and NK1.2 as described in [14] with the sole modification that the sucrose concentration in the buffer used to resuspend the membranes was raised from 400 mM to 600 mM. This modification was necessary to obtain larger membrane fragments which could readily be sedimented by centrifugation at $140\,000 \times g$ for 1 h, to free the starting material of Ni^{2+} chelating EDTA. This procedure yielded about 1–2 g of mitochondrial protein from 100 g of cells (wet weight) with a specific dNADH:HAR activity of about 1.2 μmol min^{-1} mg^{-1}.

The pellet was resuspended in 50 mM NaCl, 20 mM Na_3BO_3, pH 7.2 and adjusted to a concentration of 20 mg/ml total protein.

4000 mg mitochondrial protein were used in a typ-

ical preparation. For complete membrane protein extraction, 1 g dodecyl maltoside per gram protein was added, and 1 mM PMSF was present as protease inhibitor. The solution was stirred on ice for 5 min and then centrifuged at 140 000×*g* for 1 h. The supernatant was adjusted to 400 mM NaCl and 65 mM imidazole prior to loading onto the 50 ml Ni-NTA fast flow Sepharose column (Pharmacia) equilibrated with 65 mM imidazole, 400 mM NaCl, 0.1% dodecyl maltoside and 20 mM Na-phosphate, pH 7.2. The column was washed with 120 ml of the same buffer and tagged complex I was eluted with 150 ml of 140 mM imidazole, 400 mM NaCl, 0.1% dodecyl maltoside and 20 mM Na-phosphate, pH 7.2 at a flow rate of 160 ml/h. Fractions containing dNADH:HAR activity were combined and concentrated by centrifugation using Centricon 30 cartridges (Millipore). This pool was applied to a 21.5 mm×60 cm TSKgel G4000SW column (TosoHaas), which had been equilibrated with 100 mM NaCl, 1 mM EDTA, 25 mM Na/MOPS, pH 7.2 and 0.05% dodecyl maltoside. Chromatography was performed in a Beckman Biosys2000 FPLC system at a flow rate of 120 ml/h in the same buffer. The combined peak fractions containing dNADH:HAR activity were pooled, concentrated to 10–15 mg/ml protein as described above and stored in liquid nitrogen.

2.7. *Reconstitution of isolated complex I into proteoliposomes*

Purified *Y. lipolytica* complex I was reinserted into liposomal membranes by the detergent removal technique essentially as described in [15] and [16] using SM-2 Bio-Beads (Bio-Rad). 10 mg/ml of soybean lipid (asolectin) was sonicated in 1.6% octyl glucoside, 50 mM KCl, 20 mM Tris-Cl, pH 7.4. 200 μg of *Y. lipolytica* complex I were incubated under agitation in 1 ml of the sonicated solution for 15 min. 100 mg of Bio-Beads, pretreated as described in [17], were added to the mixture; additional 100 mg portions of Bio-Beads were added after 30 min, 90 min and 150 min of agitation. Eventually, the mixture was agitated for another 90 min to complete the detergent removal process. All incubation steps were performed at 4°C. The supernatant containing the complex I proteoliposomes was removed using a thin pipette, the Bio-Beads were washed again twice with 1 ml 50 mM KCl, 20 mM Tris-Cl, pH 7.4 and the combined supernatants were centrifuged at 100 000×*g* for 90 min. The pellet was carefully resuspended in 50 mM KCl, 20 mM Tris-Cl, pH 7.4 to a final volume of 200 μl.

3. Results and discussion

3.1. *Deletion of the chromosomal copy of the NUGM gene and complementation by gene copies encoding a his-tagged 30 kDa subunit*

The entire reading frame of the *NUGM* gene was deleted by homologous recombination using a *URA3* marked deletion allele (Fig. 1) in the diploid *Y. lipolytica* strain GB1. Replicative plasmids carrying the genomic *Sal*I fragment comprising the *NUGM* open reading frame that had been C-terminally extended by sequences encoding six histidines (pNK1.1) and six histidines plus an additional six alanine linker (pNK1.2) were used to transform this heterozygous deletion strain NK1 (Fig. 2). As a control, a plasmid carrying the unchanged *NUGM* gene was also transformed into NK1. Haploid strains carrying only a plasmid-borne copy of *NUGM* were obtained through sporulation and subsequent random spore analysis.

3.2. *Purification of his-tagged complex I*

Both constructs bearing the his tag and the his tag plus alanine linker exhibited normal expression of the *NUGM* gene. Mitochondrial membranes from

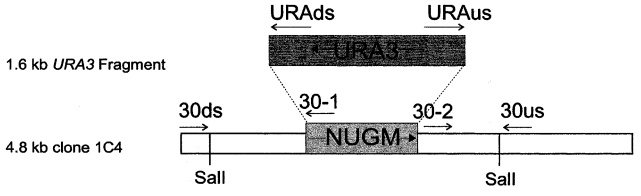

Fig. 1. Strategy for chromosomal deletion of the NUGM open reading frame. Primers 30-1 and 30-2 were used in inverse PCR to create a deletion for almost the entire *NUGM* open reading frame. A PCR product of the *URA3* gene was inserted into the gapped plasmid by blunt end ligation. A 3.0 kb *Sal*I fragment was transformed into the diploid strain GB1. Primer pairs 30ds/URAds and 30us/URAus were used to verify homologous recombination.

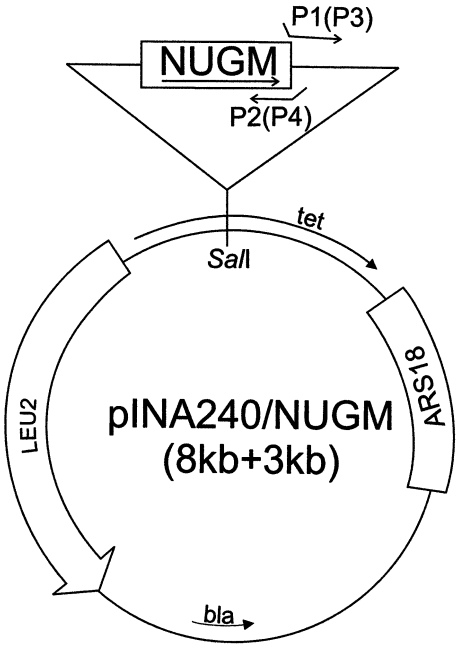

Fig. 2. Construction of expression vectors pNK1.1 and pNK1.2. pINA 240/*NUGM* was used as template for inverse PCR. Primer 1 coded for three histidines and the stop codon, primer 2 for three histidines as extensions. The self-ligated product of this PCR is referred to as pNK1.1 and was used as a template for the construction of pNK1.2. Extensions of primers 3 and 4 each coded for three alanines. bla, β-lactamase gene; *Y. lipolytica* chromosomal ARS18, ARS/CEN region (accession No. M91600); tet, tetracycline resistance gene.

all complemented deletion strains exhibited levels of dNADH:ubiquinone oxidoreductase activity similar to the parental strain (data not shown). The additional amino acids did not interfere with the assembly of the multi-subunit enzyme and no subcomplexes of complex I were detectable by two-dimensional blue-native electrophoresis (data not shown). Western blotting using anti-his tag antibody revealed that the size of the 30 kDa was increased as expected (data not shown) and that the tags were markedly stable against proteases, as apart from the addition of PMSF no special measures were needed to prevent proteolytic digestion.

Application of total mitochondrial membranes extracted with 1 g/g of dodecyl maltoside to Ni-NTA columns revealed that both tags were accessible to the resin and bound with high affinity. Experience showed that complex I from NK1.2, possessing the his tag with the six alanine linker, would bind slightly tighter to the Ni-NTA column and allowed a wash step with 65 mM imidazole while NK1.1 started to elute under these conditions (data not shown). Obviously, the six additional alanines added in front of the histidines improved accessibility of the tag to the column. Since washing with 65 mM imidazole significantly improved the purity of the preparation, NK1.2 was used and – if not stated otherwise – only results obtained with this strain will be discussed in the following.

An average of 80–90% of complex I in the total extract bound to the Ni-NTA column and eluted at 140 mM imidazole as a single peak. The pooled fractions had a specific dNADH:HAR activity of around 35 μmol min^{-1} mg^{-1} corresponding to a 20-fold enrichment of complex I by this single step. The Ni-NTA pool was concentrated and loaded onto a TSK G4000 column for further purification and removal of imidazole. The latter was also required for EPR spectroscopic analysis, as high concentrations of imidazole were found to lead to reoxidation of all iron-sulfur clusters but N2 in samples previously reduced with NADH. Complex I eluted as a symmetric from the TSKgel G4000W column (not shown). Contaminations still present after affinity chromatography could be removed by this step. With high reproducibility, the TSKgel G4000W pool exhibited a dNADH:HAR activity of around 65 μmol min^{-1} mg^{-1}, which means that complex I was enriched 70-fold as compared to mitochondrial membranes (Table 1). Overall, starting with around

Table 1
Purification of his-tagged *Y. lipolytica* complex I from strain NK1.2

Fraction	Protein		dNADH:HAR activity		Yield (%)
	mg	%	μmol min^{-1}	μmol min^{-1} mg^{-1}	
Membranes	4000	100	3540	0.9	100
Lauryl maltoside extract	1700	42	2670	1.6	76
Ni-NTA pool	43	1.1	1520	32	43
TSKgel G4000SW pool	19	0.5	1350	64	38

4000 mg of mitochondrial membranes, 18–20 mg of highly pure complex I could be isolated by a highly reproducible two step protocol (Table 1). Thus, compared to the conventional protocol [3], his tag affinity purification of complex I cuts down the preparation time by half (down to about 7 h) and yields 2–3 times more enzyme of significantly higher purity.

3.3. Properties of purified his-tagged complex I

Comparison of purified his-tagged complex I with conventionally purified [3] wild-type enzyme by tricine SDS-PAGE [18] demonstrated the much higher purity of the affinity purified enzyme and also confirmed complete assembly (Fig. 3). Less than 2% of cytochrome oxidase as very minor contaminant could be detected by UV spectroscopy. The increased size of the 30 kDa subunit also demonstrated that both variants of the his tag had remained stable in the complex during the purification procedure.

No significant differences of other features were observed in comparison to conventionally purified complex I from *Y. lipolytica* [3]: EPR spectra of the reduced iron-sulfur clusters were found to be identical (K. Zwicker et al., unpublished) and negative stain images of single particles showed a homogeneous population of L-shaped molecules (M. Radermacher et al., unpublished). These observations not only demonstrated the structural integrity of purified complex, but also confirmed the higher purity and homogeneity of *Y. lipolytica* complex I purified by affinity chromatography.

Like complex I purified from *Y. lipolytica* [3] and other sources [19,20] by conventional methods, the purified his-tagged enzyme exhibited only a very low specific dNADH:ubiquinone oxidoreductase activity of 0.1 μmol min^{-1} mg^{-1}. Already upon addition of detergent for total extraction, we observed a 2–3-fold decrease of DBQ reductase activity. However, the loss of activity was almost fully reversible by reconstitution of the purified complex into asolectin proteoliposomes (see below).

3.4. Reconstitution of complex I into proteoliposomes

Purified, his-tagged complex I was reconstituted into proteoliposomes by the detergent removal method. Using the dNADH:HAR oxidoreductase activity as a measure for complex I content we estimated that 15–20% of added complex I was recovered in the proteoliposomes. dNADH:HAR oxidoreductase activity was also used to determine the orientation of complex I in the liposomal membrane. We found that 80–90% of the NADH binding sites were accessible already in sealed proteoliposomes, the remaining complexes becoming accessible after permeabilization of the membranes by the addition of 0.1% CHAPS during the assay. This indicated that the majority of the complex I molecules were oriented inside-out when compared to mitochondria.

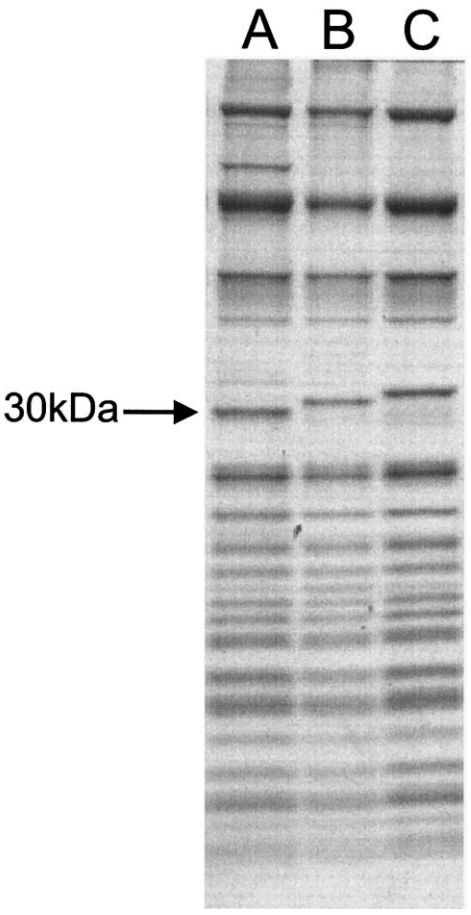

Fig. 3. SDS-PAGE of purified complex I. Tricine SDS-PAGE of purified complex I on a 16% gel was performed according to [18]. The increased size of the 30 kDa subunit (arrow) due to the addition of a his tag (lane B) and a his tag plus alanine linker (lane C) is clearly visible. Lanes: A, 7 μg of conventionally purified complex I [3]; B, 5 μg of affinity purified complex I from strain NK1.1; C, 7 μg of affinity purified complex I from strain NK1.2.

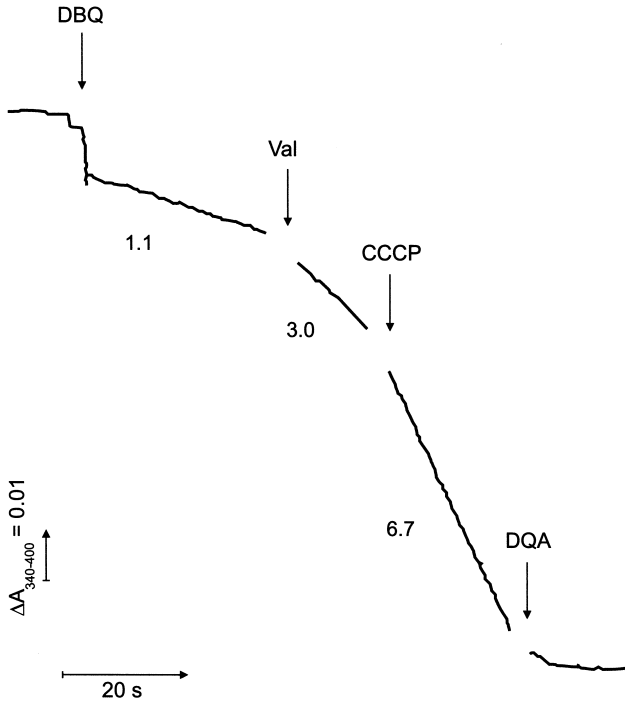

Fig. 4. dNADH:ubiquinone oxidoreductase activity of complex I purified from strain NK1.2 reconstituted into asolectin proteoliposomes. dNADH:DBQ oxidoreductase activities of 3.3 μg complex I in proteoliposomes were measured in the presence of saturating concentrations of substrates (100 μM dNADH, 60 μM DBQ). The reaction was started by the addition of DBQ. 2 μM valinomycin (Val), 2 μM CCCP and 2 μM DQA were added as indicated. Rates are indicated as specific activities (μmol min^{-1} mg^{-1}).

dNADH:ubiquinone oxidoreductase activity of complex I in proteoliposomes was stimulated 6–7-fold upon uncoupling with 2 μM valinomycin and 2 μM CCCP (Fig. 4). As this indicated the generation of a proton-motive force, we conclude that purified tagged complex I was capable of translocating protons across the liposomal membrane.

When soybean asolectin was used for reconstitution, the dNADH:ubiquinone oxidoreductase turnover number recovered essentially to the value calculated for mitochondrial membranes: reconstitution into asolectin proteoliposomes increased the specific dNADH:ubiquinone oxidoreductase activity more than 60-fold from 0.1 μmol min^{-1} mg^{-1} for isolated complex I to 6.7 μmol min^{-1} mg^{-1} in uncoupled, permeabilized proteoliposomes (Fig. 4), corresponding to a turnover number of about 100 s^{-1}. This activity could be inhibited completely by the addition of 2 μM DQA during the assay. It should be noted that almost no stimulation of DBQ reductase activity was observed when egg yolk phospholipid rather than asolectin was used for reconstitution.

4. Conclusions

Attaching a his tag and a linker of six alanine residues to the C-terminus of the 30 kDa subunit of complex I for the first time allowed affinity purification of this membrane bound 900 kDa multiprotein complex. The high cell yield of *Y. lipolytica*, its constitutively high content of mitochondria and a complex I recovery of almost 40% by the purification protocol described here result in an overall yield of 40–80 mg of a fully assembled, highly pure and homogeneous preparation of this enzyme complex from 10 l of overnight culture. Following reconstitution into proteoliposomes the purified enzyme exhibits full, inhibitor sensitive activity and is capable of generating a proton-motive force. Thus, affinity purified complex I seems very well suited for crystallization attempts aimed at eventually resolving the molecular structure of mitochondrial complex I.

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